Mechanism of Platelet-Derived Growth Factor-Dependent Caveolin-1 Phosphorylation: Relationship to Sterol Binding and the Role of Serine-80†

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ABSTRACT: In human vascular smooth muscle cells, inhibitors of protein kinase C activity reduced serine phosphorylation of caveolin-1 and increased sterol binding by this protein. This was measured after immunoprecipitation of caveolin-1 from cells labeled with tritiated cholesterol or the photoactivable cholesterol analogue FCBP [Fielding et al. (2002) *Biochemistry 41*, 4929−4937]. At the same time cellular sterol efflux was inhibited. Mutagenesis within a caveolin-1 central domain (residues 80−104) suggested a major role for serine-80 in mediating both of these effects. To perturb sterol binding, platelet-derived growth factor was added to the cells, leading to a transient loss of caveolin-1-associated sterol. Under these conditions, sterol efflux was stimulated, and caveolin-1 phosphorylation at tyrosine$_{14}$, assayed with a selective antibody, was substantially increased above baseline levels. These changes were also blocked by inhibitors of protein kinase C activity. Selective inhibitors of the platelet-derived growth factor receptor and downstream kinases were used to show that loss of sterol from caveolin-1 preceded tyrosine phosphorylation, but relipidation was dependent on phosphotyrosine hydrolysis.

Caveolae are cell surface microdomains, enriched in free cholesterol (FC).[1] They are abundant in terminally differentiated peripheral cells including vascular smooth muscle cells (SMC), endothelial cells, and adipocytes (*1*−*3*). Many receptor kinases, as well as nonreceptor kinases and nonkinase signal-related proteins (linkers), are located in caveolae. The platelet-derived growth factor receptor (PDGFR) protein is among those best characterized (*4*, *5*). Platelet-derived growth factor (PDGF) binding promotes the dimerization and autophosphorylation of PDGFR. The modified receptor protein in turn activates linkers (such as Shc) and other kinases including Ras, phosphatidylinositol 3-kinase (PI3K), protein kinase Cα (PKCα), and c-Src family proteins (*6*−*9*). Phospho-PDGFR (p-PDGFR) is a substrate for a low molecular weight phosphotyrosine phosphatase (LMW-PTP) in caveolae (*10*). The equilibrium between the PDGFR kinase and phosphatase pathways regulates the magnitude and duration of signaling by PDGF.

The major structural protein of caveolae, caveolin-1, serves as a scaffold for signaling complexes, including those containing PDGFR, via a central domain. Caveolin-1 may be phosphorylated at S_{80} and possibly other serine residues (*11*, *12*). Phosphorylation at tyrosine-14 (Y_{14}), which is low in quiescent cells, is stimulated in response to insulin, insulin-related growth factor, and vascular endothelial growth factor (*13*−*15*). Like p-PDGFR, p(Y_{14})-caveolin-1 is a substrate for LMW-PTP (*16*), but in contrast to PDGFR, the functional role, if any, of caveolin-1 phosphorylation has not yet been identified.

In addition to signal-related proteins, caveolin-1 binds FC (*17*, *18*), and roles for caveolin-1 in intracellular FC transport and FC efflux from the cell have been described (*19*, *20*). The signaling and FC homeostatic functions of caveolin-1 appear to be codependent. During the response of SMC to PDGF, FC association with caveolin-1 was markedly reduced. Conversely, when FC efflux from the cell was increased by the presence of an extracellular FC acceptor, apolipoprotein A-1 (apo A-1), protein kinase activity was amplified (*18*). Effects of FC on signal transduction from caveolae have also been reported for other signaling pathways (*15*, *21*).

Among nonreceptor kinases in caveolae PKC, which phosphorylates serine and threonine (S, T) residues, has been implicated as a regulator of caveolin-1 phosphoserine levels in nonstimulated cells (*11*). The activities of PKC and PDGFR are linked by several pathways in PDGF-activated cells, for example that mediated by the protein phosphatase SHP-2, which is activated by PKC to stimulate c-Src (*22*). PKC activators increased FC efflux from SMC (*23*), but the mechanism of this effect is still undefined. In the present study, the relationships between PDGF, PKC, caveolin-1, and FC efflux have been explored. The data obtained provide

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[1] Abbreviations: apo A-1, apolipoprotein A-1; FC, free cholesterol; FCBP, 22-(*p*-benzoylphenoxy)-23,24-bisnorcholan-5-en-3*β*-ol; LMW-PTP, low molecular weight phosphotyrosine phosphatase; NGIC-I, nonglycosidic indolocarbazole I; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PKC, protein kinase C; SMC, smooth muscle cells.

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new evidence for interdependent roles for caveolin in signaling and FC homeostasis, identify intermediates of signal transduction leading to FC efflux from caveolae, and indicate that the level of FC associated with caveolin-1 is a component of cell FC responsive to both PDGFR and PKC.

EXPERIMENTAL PROCEDURES

Materials. Bisindolylmethanone, an inhibitor of PDGFR autophosphorylation (*24*), was synthesized from *N*-phenylsulfonylindole and *N*-phenylsulfonylindole-2-carboxyaldehyde (*25*, *26*). Other inhibitors were from Calbiochem, San Diego, CA, and were used as described by the supplier. Polyclonal anti-caveolin-1 antibody and an antibody (N-20) recognizing its nonphosphorylated N-terminal domain were from BD Biosciences/Transduction Laboratories, Lexington, KY, and from Santa Cruz Biotechnology, respectively; p(Y_{14})-caveolin-1 antibody was from Cell Signaling Technology, Beverley, MA. Anti-phosphoserine antibody was from Sigma, St. Louis, MO. Human recombinant PDGF-BB was from R & D Systems, Minneapolis, MN. [1,2-^{3}H]FC (50−55 Ci/mmol) was from Pharmacia-NEN (Boston, MA). A ^{3}H-labeled derivative of photoactivable benzophenone-containing FC (FCBP) was synthesized as described (*18*) at Pharmacia-NEN. Full-length human caveolin-1 cDNA (*27*) was modified by insertion of a FLAG-tagged C-terminus to the coding region. Mutants of this construct were generated using Quik-Change mutagenesis kits (Stratagene, La Jolla, CA). Polyclonal antibody to the C-terminal FLAG epitope was from Sigma. Glucose oxidase from *Aspergillus niger* (20 units mg^{-1}) was from Calbiochem.

Cell Culture. Primary human arterial SMC were obtained from Cambrex, Walkersville, MD. The cells were routinely cultured at 37 °C in basal medium + 5% (v/v) fetal bovine serum (FBS) (*18*). For individual experiments, SMC were plated in 3.5 cm plastic dishes. On reaching 70−80% confluency (3−5 days) the cells were serum-starved (36−48 h) in medium containing 1 mg mL^{-1} high molecular weight dextran (T-500, Pharmacia) to upregulate PDGFR expression. The effects of added PDGF (100 ng mL^{-1}) were determined over a time course of 2.5−45 min. In some experiments, the cells had been preequilibrated with [1,2-^{3}H]FC or [^{3}H]FCBP for 24−48 h prior to serum starvation.

Immunofluorescence Microscopy. SMC were rinsed and fixed in 3% (w/v) formaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature and then permeabilized with 0.1% Triton X-100 (15 min). Nonspecific binding was blocked with 5% donkey serum−PBS (30 min). The fixed cells were incubated with polyclonal antibody to the caveolin-1, with antibody to Y_{14}-phosphorylated N-terminal peptide, or to anti-C-terminal FLAG antibody. Staining, distribution, and intensity were evaluated with a computerized Nikon Eclipse TS 100 microscope.

Immunoprecipitation of Caveolin-Associated Lipids. Caveolin-associated sterol was assayed using either of two methods described previously (*18*). Briefly, SMC labeled with [^{3}H]FC were incubated in 50 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA (pH 7.4) containing 1% (w/v) Nonidet P-40 (*28*). The cells were collected and sheared using a 27G needle. Following centrifugation (10 min, 12000*g*) caveolin polyclonal antibody was added to the postnuclear supernatant, and the mixture was incubated for 2−4 h at 4 °C. The protein G−agarose covalent complex was added, and incubation was continued for a further 1.5 h. The beads were pelleted by centrifugation through 10% (w/v) sucrose. After being washed under the same conditions, the beads were extracted with liquid scintillation cocktail, and the [^{3}H]FC label was quantitated. Alternatively, [^{3}H]FCBP-labeled cells were UV irradiated (365 nm, 15−60 min, 0 °C) to cross-link the sterol benzophenone group to the α-carbon of the adjacent protein. Immunoprecipitation with anti-caveolin or anti-FLAG antibody was carried out as described above. Purified complexes were extracted with sample buffer containing SDS and fractionated by PAGE. The level of caveolin-associated ^{3}H-label was determined by liquid scintillation spectrometry.

Electrophoresis and Immunoblotting. Cell lysates or immunoprecipitated protein complexes were solubilized in sample buffer plus 1.0 M 2-mercaptoethanol and then fractionated by PAGE. Protein in cell lysates was determined using bicinchoninic acid (Pierce, Rockford, IL). Following electrotransfer to nitrocellulose sheets (0.2 μm pore) blotting was carried out with antibodies to caveolin or to intermediate proteins of PDGF-dependent signaling pathways. Protein bands were visualized with Supersignal chemiluminescent substrate (Pierce). Signal intensity was measured with a Molecular Dynamics scanning densitometer. Caveolin-1 is present as two isoforms reflecting, at least in part, the contribution of alternative translational start sites. The shorter (β) isoform lacks the first 31 amino acids of the longer (α) isoform, including the tyrosine residue at position 14. In the present experiments, the β-isoform was only a minor proportion (6 ± 2%) of total caveolin-1, as determined by Western blotting with polyclonal anti-caveolin-1 antibody.

Caveolin Mutagenesis, Transfection, and Expression in SMC. Wild-type caveolin-1-FLAG cDNA was modified by alanine mutagenesis of serine residues (S80A, S88A, S104A) spanning the central domain implicated in signal protein binding (*2*). S_{37}, identified previously as part of a PKC consensus site (*29*), and Y_{14}, phosphorylated by several protein growth factors, were modified in the same way. All DNA sequences were confirmed by the UCSF Cancer Center Core Facility. Transfection was carried out with an Amaxa Nucleofector (Amaxa Biosystems) according to the manufacturer's instructions for SMC. Transfection efficiency, determined by immunofluorescence, was 50−65%. The proportion of FLAG-tagged caveolin-1 to total caveolin-1 was 6−25% in different transfections. The distribution of FLAG-tagged caveolin-1 by immunofluorescence was determined with anti-FLAG antibody. Transfected cells were cultured (36 h) in [^{3}H]FCBP-labeled medium. Immunoprecipitation, solubilization, and SDS gel electrophoresis were carried out as described above. The ^{3}H content of the FLAG-tagged component was determined, normalized to caveolin-1 mass. There was no significant difference in FC efflux rates, determined as described below, between transfected and sham-transfected wild-type cells. This indicates that FC homeostasis was substantially normal in FLAG-transfected SMC.

FC Efflux. FC efflux was expressed as the rate of transfer of cellular [^{3}H]FC from fully equilibrated SMC to apo A-1−phospholipid complexes generated by preincubation with human skin fibroblasts. Briefly, apo A-1 (10 μg mL^{-1}) was

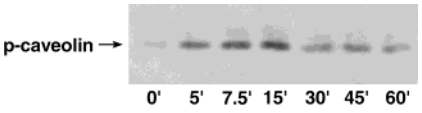

FIGURE 1: Response of p(Y_{14})-caveolin-1 levels in quiescent SMC to PDGF (100 ng mL^{-1}) as a function of time. The level of p(Y_{14})-caveolin-1 was assayed in cell homogenates using specific anti-p(Y_{14})-caveolin-1 antibody. The decrease in p-caveolin-1 levels between 15 and 30 min was 88 ± 6% ($n = 5$).

incubated with cells for 2−4 h at 18 °C. Acceptor particles, analyzed by nondenaturing PAGE, formed a single population (6.7 nm diameter). Their phospholipid content was 8.2 ± 1.0% (w/w). FC was <0.5% (w/w). The fibroblast conditioned medium was transferred to [^{3}H]FC-labeled SMC, incubated under the same conditions, centrifuged (10 min, 10000 rpm, 4 °C) to remove any floating cells, and then analyzed for radioactivity. Studies of PDGF-stimulated FC efflux were carried out at 37 °C. Studies of nonstimulated (basal) rates of FC efflux were carried out at 18 °C as previously described (*18*) to inhibit refilling of caveolae from intracellular FC pools. In both cases, FC efflux was linear as a function of time.

RESULTS

p(Y_{14})-Caveolin-1 Synthesis Mediated by PDGF. Following addition of PDGF (100 ng mL^{-1}) to SMC, p(Y_{14})-caveolin-1 levels, which initially were very low, increased to reach a maximum after 7.5−15 min that was 10−15-fold above background. By 30 min, these levels had significantly decreased (Figure 1). The response was similar if PDGF remained in contact with the cells or if it was removed after 2 min. This indicates that, after activation, SMC entered a nonresponsive phase. After 30−45 min in the absence of PDGF, SMC were once more responsive to PDGF (data not shown).

Caveolin-1 immunofluorescence in nonstimulated SMC was present mainly at the cell surface (Figure 2), consistent with previous reports (*31*). In response to PDGF (7.5 min) p(Y_{14})-caveolin-1 immunofluorescence intensified at the periphery. Its subsequent decrease was associated with reappearance of unmodified caveolin-1 at the cell surface. FLAG immunofluorescence seen in cells transfected with caveolin-1-FLAG cDNA was similarly localized. Access of this protein to the cell surface was confirmed by the appearance of FLAG-tagged p-caveolin after the exposure of these cells to PDGF.

Mechanism of p(Y_{14})-Caveolin-1 Formation. SMC were preincubated with inhibitors of PDGFR or c-Src family kinase activities. Caveolin-1 p(Y_{14}) phosphorylation in response to PDGF was comparably reduced by both classes of inhibitor. For example, AG1296, which blocks PDGFR autophosphorylation (*32*), and PP2, which inhibits downstream tyrosine phosphorylation (*33*), both reduced the p-caveolin-1 response to PDGF ~90% compared to control levels (Figure 3). This showed that the action of PDGF in promoting caveolin-1 Y_{14} phosphorylation was mediated by c-Src or a related kinase. In view of the role reported for PKC in cellular FC homeostasis (*23*) and the link previously identified between growth factors and FC binding in caveolae (*18*), PKC inhibitors were tested for their effects on p-caveolin-1 levels. The nonglycosidic indolocabazole NGIC-I, a potent inhibitor of PKC family kinases (*34*, *35*), and myristoylated PKC_{20-28} inhibitor peptide (an antagonist of the PKCα/β activity) (25 μM) (*36*) inhibited p-caveolin formation to 0.04 ± 0.05 and 0.08 ± 0.01 of uninhibited levels, respectively, comparable to the effects of AG1296 and PP2. In contrast, inhibitors of the phospholipase C, phosphatidylinositol 3-kinase, and MAP kinase pathways and pertussis toxin (an activator of G-protein-dependent signaling) had no effect under similar conditions. Neither dioleyl-

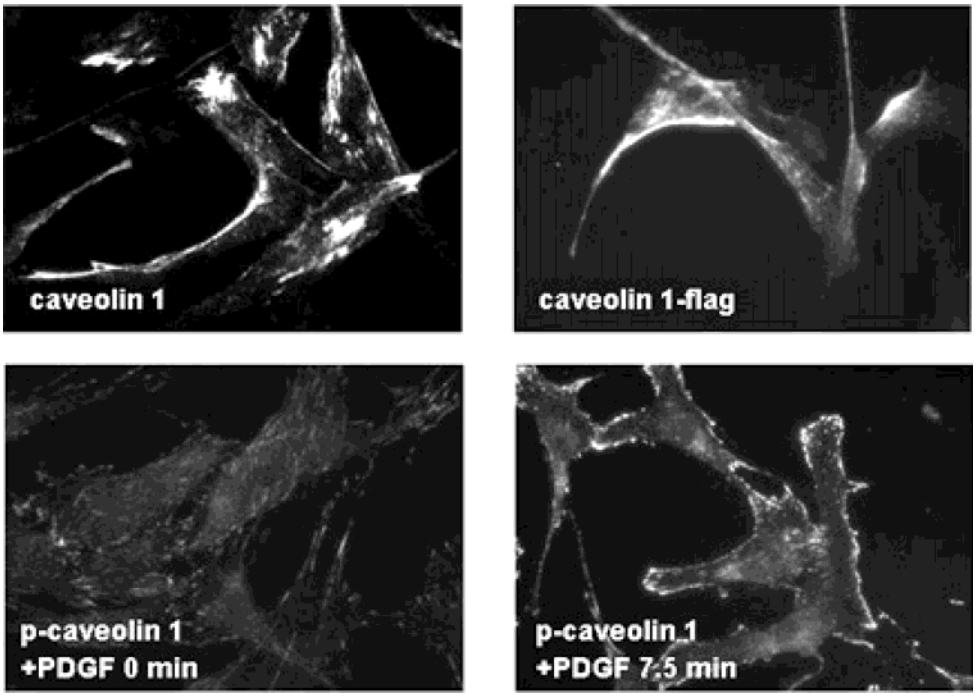

FIGURE 2: Caveolin-1 and p(Y_{14})-caveolin-1 immunofluorescence of wild-type SMC. Upper left: nonstimulated cells, stained with antibody to nonphosphorylated caveolin-1 N-terminal peptide. Upper right: SMC transfected with caveolin-1-FLAG cDNA, stained with anti-FLAG antibody. Lower left: SMC, stained with antibody to p(Y_{14})-caveolin-1 N-terminal antibody. Lower right: SMC following the addition of PDGF, stained with the same antibody.

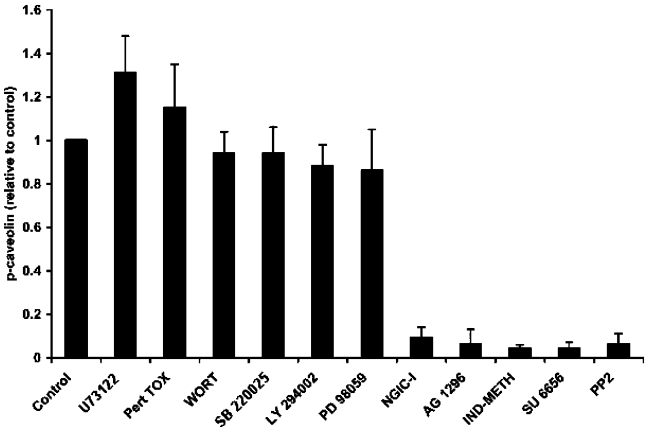

FIGURE 3: Effects of regulators of signal transduction on p(Y_{14})-caveolin-1 levels in SMC preincubated (7.5 min) with PDGF. Inhibitors were added 20 min prior to the addition of PDGF at the following concentrations: WORT (wortmannin), 25 nM; PP2, 50 nM; NGIC-I, U73122, SB220025, and SU6656, 200 nM; IND-METH (indolylmethanone), AG1296, PD98059, and LY294002, 2 μM. Pertussis toxin (Pert TOX), an activator of G-protein-catalyzed signaling, was added to a final concentration of 100 ng mL^{-1}. After a further 7.5 min, the cells were extracted with SDS buffer, fractionated by electrophoresis, transferred to nitrocellulose, and blotted with anti-p(Y_{14})-caveolin-1 antibody. The data were quantitated by densitometry as described under Experimental Procedures. Levels are expressed relative to those measured in the absence of inhibitor (1.0). Except as indicated in this and the following figures, the data shown represent the mean ± 1 SD of three to five independent experiments.

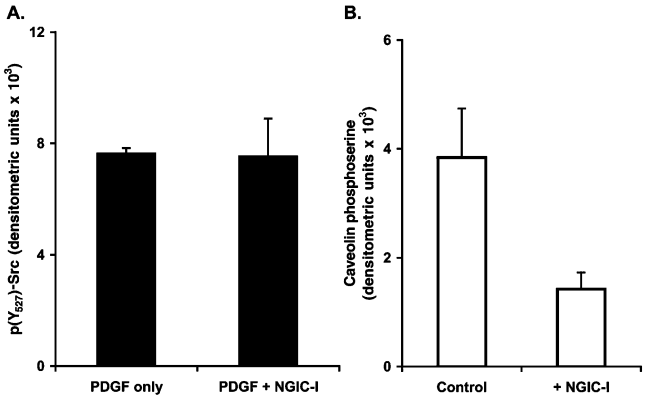

FIGURE 4: (A) Effect of NGIC-I on p(Y_{527})-Src levels 15 min following exposure to PDGF. SMC were incubated for 20 min with or without NGIC-I (100 nM) prior to the addition of PDGF (100 ng mL^{-1}). Levels of p-Src in whole cell extracts were determined by immunoblotting. (B). Effect of NGIC-I on caveolin-1 phosphoserine levels. After precipitation with polyclonal anti-caveolin-1 antibody, caveolin-1 from nonstimulated SMC was fractionated by SDS electrophoresis, analyzed by immunoblotting with polyclonal phosphoserine antibody, and quantified by densitometry as described under Experimental Procedures.

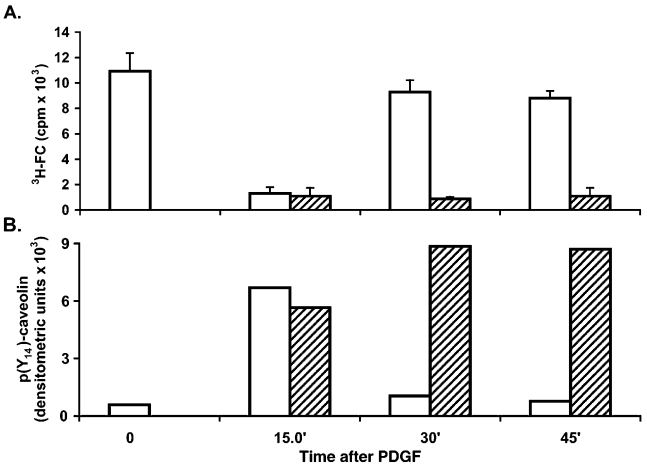

FIGURE 5: Effects of glucose oxidase on p(Y_{14})-caveolin-1 and caveolin-1-associated FC levels following the addition of PDGF (100 ng mL^{-1}). (A) Caveolin-1-associated [^{3}H]FC as a function of time following the addition of PDGF. Immunoprecipitation of extracts from [^{3}H]FC-labeled SMC was carried out with rabbit polyclonal caveolin-1 antibody and complexed to protein G−agarose. Key: open bars, without glucose oxidase; hatched bars, in the presence of glucose oxidase (1.0 μg mL^{-1}, added 5 min prior to addition of growth factor). Values shown are means ± 1 SD (n = 3). (B) p(Y_{14})-caveolin levels in SMC extracts as a function of time after the addition of PDGF. Key: open bars, without glucose oxidase; hatched bars, plus glucose oxidase. Values shown are means from two identical experiments, whose values differed <5%.

glycerol nor phorbol ester increased p-caveolin above basal levels under these conditions. This finding suggested that PKC was constitutively activated in caveolae.

PKC might mediate p(Y_{14})-caveolin-1 synthesis by regulating the phosphorylation of c-Src whose activity is negatively regulated via phosphorylation at Y_{527} (*37*). Alternatively, PKC could phosphorylate caveolin-1 at serine or threonine residues, directly or via an ancillary kinase (*11*) to influence the substrate properties of caveolin-1 with tyrosine kinases.

p(Y_{527})-Src levels, initially raised, were transiently decreased 10−20 min following the addition of PDGF. However, NGIC-I had no significant effect on c-Src activation measured with specific antibody to this protein species (Figure 4A). On the other hand, NGIC-I halved phosphoserine levels in immunoprecipitates of caveolin-1 (Figure 4B), a reduction consistent with previous data (*11*). These results suggest that the effects of PKC inhibitors on p-caveolin-1 synthesis might be mediated at the level of caveolin phosphoserine content.

Effects of PDGFR and PKC Activities on Caveolin-1-Associated FC. In addition to its effects on caveolin-1 phosphorylation, PDGF-mediated signal transduction was associated with a significant reduction in caveolin-1-associated FC and a stimulation of cellular FC efflux (*18*). The time course of these events was compared. Fifteen minutes following the addition of PDGF, >80% of caveolin-associated FC had been lost. After 30 min, however, FC had returned almost to its original level (Figure 5A). Glucose oxidase generates hydrogen peroxide in reaction with glucose to inhibit LMW-PTP activity (*38*). Under these conditions, the recovery of caveolin-associated sterol levels was found to be prevented.

These changes contrast with the pattern of p(Y_{14})-caveolin-1 levels seen in response to the same factors (Figure 5B). p-Caveolin-1 levels, which initially were low, were increased after the addition of PDGF, but when glucose oxidase was absent, these levels quickly declined. When glucose oxidase was present, high levels of p-caveolin-1 were maintained. Together, these data suggested that loss of caveolin-associated sterol in response to PDGF might be linked to phosphorylation at Y_{14}.

To study this relationship further, [^{3}H]FC-labeled SMC were incubated with PDGF for 7.5 min. The label associated with the phosphorylated and nonphosphorylated caveolin

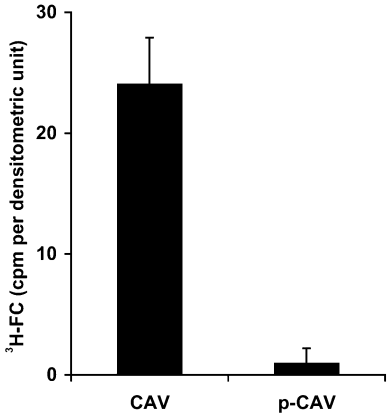

FIGURE 6: [^{3}H]FC content of caveolin immunoprecipitated with either anti-p-caveolin-1 N-terminal peptide antibody or unmodified N-terminal peptide antibody 7.5 min following the addition of PDGF (100 ng mL^{-1}). The label has been normalized to caveolin-1 mass, determined from Western blots.

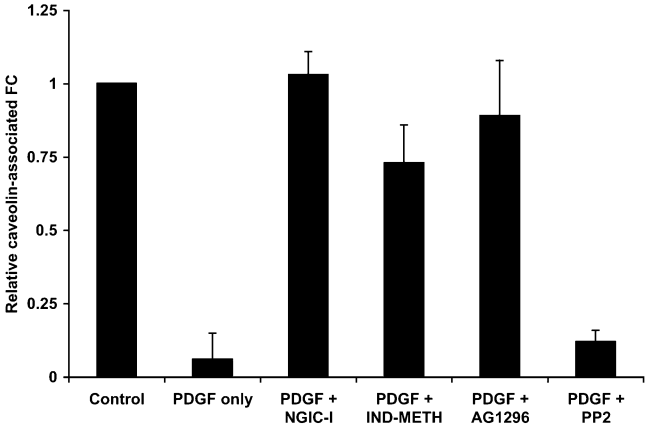

FIGURE 7: Effects of signaling inhibitors on caveolin-1-associated FC. Experiments were carried out using [^{3}H]FC-equilibrated SMC, incubated (20 min) in the presence or absence of AG1296, bisindolylmethanone (IND-METH), PP2, or NGIC-I under the conditions described in the legend to Figure 3. The data represent caveolin-1-associated [^{3}H]FC, expressed relative to control (1.0), 15 min after the addition of PDGF (100 ng mL^{-1}).

fractions was determined by separate immunoprecipitations, using antibody specific either for the N-terminal peptide of unmodified caveolin or for the p(Y_{14}) N-terminal caveolin peptide. While unmodified caveolin-1 was associated with substantial levels of [^{3}H]FC, p-caveolin was strongly FC-depleted (Figure 6). These data suggest that the p(Y_{14}) phosphorylation of caveolin reduces its ability to bind FC.

To determine if Y_{14} phosphorylation was the cause or result of loss of FC from caveolin-1, the effect of inhibitors of PDGFR, c-Src, and PKC activities on [^{3}H]FC binding was measured. SMC pretreated with inhibitors of PDGFR autophosphorylation (AG1296, bisindolylmethanone) or NGIC-I were resistant to the loss of caveolar FC otherwise induced by PDGF-mediated signaling (Figure 7). In contrast, PP2, which inhibits c-Src activity, had no effect on the depletion of FC from caveolin-1. In other words, loss of FC appeared to be a precursor of and not a product of p(Y_{14})-caveolin-1 formation. In addition, this result suggested that the effect on FC was mediated after the activation of PDGFR but before the activation of c-Src.

FC Efflux and the Loss of Caveolar FC. PDGF-activated signaling from caveolae in SMC was associated with a

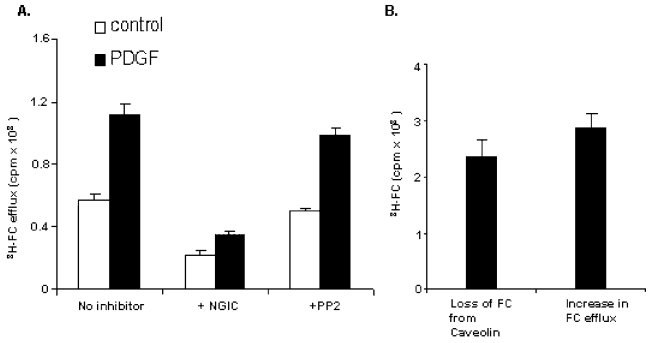

FIGURE 8: Relationship between FC efflux and caveolin-1-associated FC levels. (A) FC efflux was determined as the rate of appearance of label efflux in the extracellular medium at 37 °C from [^{3}H]FC-labeled SMC to cell-generated apo A-1−phospholipid complexes (*30*). Efflux in the presence of PDGF (100 ng mL^{-1}) was determined over the interval 5−20 min following the addition of growth factor. Where indicated, NGIC-I or PP2 (Figure 3) was added 20 min before. (B) Relationship between FC efflux and loss of caveolin-1-associated FC in [^{3}H]FC-labeled SMC. Incubation was carried out over 4 h at 18 °C into the medium described above. The medium was collected and assayed for [^{3}H]FC label. The cells were cooled on ice, solubilized, and incubated at 0 °C (90 min) with polyclonal anti-caveolin-1 antibody and for 90 min with protein G−agarose, as described under Experimental Procedures. After centrifugation and washing, [^{3}H]FC in the precipitate was determined. Caveolin-1-associated FC was 1.9 ± 0.2% of total cell label.

stimulation of FC efflux (*18*). To further define the mechanism involved, the effects of inhibitors of c-Src and PKC on basal and PDGF-activated FC efflux were determined at 37 °C. In the presence or absence of PDGF, NGIC-I significantly reduced FC efflux (Figure 8A). In contrast, but consistent with its effect on caveolin-associated FC (Figure 7), PP2 was without effect. A parallel pattern of inhibition was seen when the stimulation of FC efflux following addition of PDGF was assayed. Over 15 min, addition of PDGF was associated with a 2-fold increase in FC efflux, which was comparable to the 2.6-fold increase under the same conditions reported earlier (*18*). NGIC-I reduced efflux by 75%, below the rate observed in the absence of PDGF. In contrast, PP2 had only a small effect on PDGF-stimulated efflux (−12%) that did not reach significance. Finally, during incubation in the absence of PDGF, loss of caveolin-1-associated FC was accompanied by an equivalent increase in FC efflux (Figure 8B). The loss of caveolin-1-associated FC in these experiments following exposure to PDGF was 1.9 ± 0.2% of total cell label ($n = 4$).

Activity of FLAG-Tagged Caveolin-1 Mutant Proteins in SMC. The data above seem most consistent with a model in which the ability of PDGF to promote signal transduction [measured in terms of the c-Src-dependent p(Y_{14})-caveolin synthesis] was regulated by the FC binding status of caveolin. That, in turn, was influenced by phosphoserine levels within the central domain of the protein, while phosphorylation of signaling proteins induced by PDGF would further stimulate the displacement of FC from caveolin-1. If this model were correct, substitution of the caveolin serine residues responsible would increase FC binding under basal conditions. If, as suggested above, FC binding inhibited p(Y_{14})-caveolin formation, then such mutant proteins might also be defective in their response to PDGF.

Relatively high levels of caveolae are present in SMC. This indicates that all of the factors needed to organize

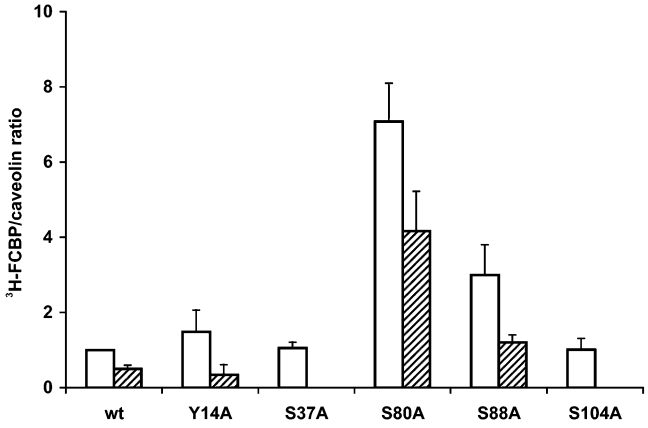

FIGURE 9: Effect of mutation on caveolin-1 sterol binding in SMC transfected with FLAG-tagged caveolin-1 and equilibrated with [^{3}H]FCBP. The cells were incubated (15 min) with PDGF prior to processing. After UV cross-linking (15 min, 0 °C) and immunoprecipitation with anti-FLAG antibody, each lipid−protein complex was analyzed by electrophoresis, immunoblotting, and liquid scintillation spectrometry. The lipid label was normalized to FLAG-caveolin-1 levels, determined by densitometry, for each transfection. The data are expressed relative to that assayed in cells transfected with wild-type FLAG-caveolin-1 cDNA in the absence of PDGF (1.0). The effect of PDGF was determined on sterol binding to mutant caveolin-1 when this differed significantly from wild type. Key: open bars, in the absence of PDGF; hatched bars, in the presence of PDGF (100 ng mL^{-1}).

caveolin into structural complexes at the cell surface are present. On the other hand, the influence of mutant caveolins on sterol binding would be diluted by the presence of the endogenous protein. To circumvent this effect, wild-type or mutant FLAG-tagged caveolins were expressed in [^{3}H]FCBP-labeled SMC. Our previous study showed this photoactivable analogue to fully equilibrate with FC in these cells (*18*). After cross-linking and immunoprecipitation of transfection products with FLAG antibody, levels of labeled sterol covalently cross-linked to caveolin-1-FLAG were determined.

Four serine residues (S_{37}, and S_{80}, S_{88} and S_{104}) were individually modified in these studies. Mutation at S_{80} led to a 7.1-fold increase in [^{3}H]FCBP cross-linked to caveolin-1 ($n = 4$, $p < 0.02$) (Figure 9). Mutation at S_{88} had a smaller (3-fold) effect while mutation of Y_{14}, S_{37}, and S_{104} had no effect on sterol binding.

The effects of mutation on the response to PDGF were determined (Figure 9). PDGF reduced the level of [^{3}H]FCBP cross-linked to (S80A)caveolin-1, but it still remained substantially (4.2-fold) higher than baseline. These data suggested that while FC can be displaced by the action of PDGF, in the S_{80} mutant it remained more tightly bound than to the wild-type protein under the same conditions. Of particular interest, the [^{3}H]FCBP content of Y14A mutant caveolin, initially within normal limits, decreased 3-fold in the presence of PDGF, even though this mutant protein lacked the Y_{14} phosphorylation site. This observation is consistent with the hypothesis that loss of sterol preceded and was independent of p(Y_{14})-caveolin formation.

If NGIC-I mediated its effects in the same way as the S_{80} mutation, by increasing sterol binding as phosphoserine levels were reduced, this inhibitor would also increase [^{3}H]FCBP label cross-linked to caveolin-1 in unstimulated cells. Consistent with this prediction, a 2.7-fold higher level of label was found in inhibited than in control cells ($n = 10$, $p < 0.01$). In addition, FLAG-tagged p(Y_{14})-caveolin-1 formation should be reduced in S80A mutant cells. This prediction was also shown experimentally (Figure 10). The increase in FLAG-precipitable p-caveolin-1 generated in cells transfected with wild-type caveolin in these experiments also confirms that it is at the cell surface.

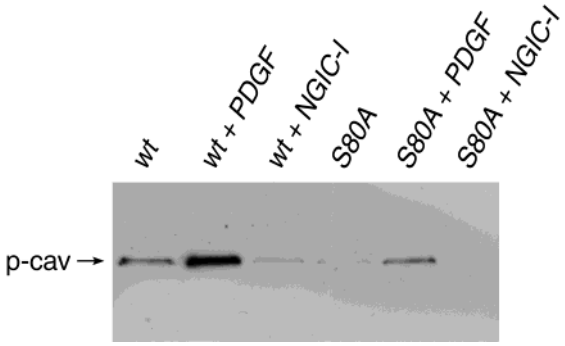

FIGURE 10: Effects of NGIC on FLAG-caveolin-1 Y_{14} phosphorylation. SMC were transfected with either wild-type or mutant (S80A) caveolin-1 cDNA. p(Y_{14})-caveolin-1 levels were measured before and after incubation (20 min) with NGIC-I (100 nM) and before and after (7.5 min) the addition of PDGF (100 ng mL^{-1}). p(Y_{14})-caveolin-1 levels in precipitates obtained using anti-FLAG antibody were determined by Western blotting.

NGIC reduced phosphoserine levels in FLAG-tagged wild-type caveolin-1 by 51 ± 9%, comparable to the reduction reported for the native protein (Figure 4B). NGIC decreased phosphoserine in FLAG-tagged S88A caveolin by 40 ± 4%. In contrast, phosphoserine in the FLAG-tagged S80A protein was not further reduced under the same conditions (+3 ± 3%) ($n = 3$). These data are consistent with a model in which S_{80} in wild-type caveolin-1, which is predominantly at the cell surface (Figure 2), is phosphorylated in SMC.

DISCUSSION

Dependence of signaling from caveolae on FC levels is now widely recognized (*18*, *21*). Nevertheless, relatively little information is available concerning how the FC content of these membrane domains is regulated. During PDGF-dependent signal transduction, loss of FC from caveolae in SMC was coupled to a stimulation of kinase activities (*18*). In the present study the relationship between caveolin-1-associated sterol levels and the PDGF-dependent tyrosine phosphorylation of caveolin-1 was examined. In particular, we asked if changes in phosphorylation could explain the differences in sterol content measured in the course of signal transduction. Since only the major (α) isoform of caveolin-1 can be phosphorylated at Y_{14}, our study was limited to this case.

Role of S_{80} in Sterol Binding. Our data suggest that S_{80} plays a major part in determining the level of sterol bound to caveolin-1. In nontransfected cells, the PKC inhibitor NGIC-I both reduced caveolin phosphoserine levels and increased caveolin-1-bound sterol in [^{3}H]FCBP-labeled SMC. In transfected cells, mutant (S80A)caveolin-1 bound significantly more sterol than the wild-type protein, and this result was not modified by NGIC-I. Together, these data support an important role for S_{80} phosphorylation in regulating sterol binding by caveolin-1, and they suggest that the effect of NGIC is mediated at least in part at this site. Sowa et al. (*39*) found that phosphorylation of serine residues in

caveolin-2 favored its location at the cell surface, as suggested here for caveolin-1. This conclusion differs from that of Schlegel et al. (*12*) based on the diversion of the S80E mutant protein (a model for serine phosphorylation) in rat Ar42J (pancreas-derived) cells into the secretory pathway. However, this mutant was for undetermined reasons also palmitoyl defective, which may modify the distribution of caveolin-1 from the cell surface (*40*). Although further research will be needed, the balance of evidence suggests that, at least in SMC, wild-type caveolin-1 is normally phosphorylated at S_{80}.

The caveolin sequence -GTHS_{80}FDG- and the PKC consensus sequence (R/KxS/TxR/K, where x is any amino acid) are quite dissimilar. As a result, it seems most likely that the effect of PKC is mediated via another serine/threonine-specific kinase. The consensus sequences recognized by Ca^{2+}/calmodulin kinase II and casein kinase 2 (CK2), requiring one or more acidic residues (D, E), show similarity to that surrounding S_{80} in caveolin. A CK2-like kinase was identified earlier in caveolae (*41*). Further research will be needed to fully determine the role of these kinases, if any, in regulating caveolin-1 phosphoserine levels. Our data do not suggest that S_{37} is functional in reaction with PKC to regulate caveolin-1 sterol binding.

On the basis of a consensus sequence present in other proteins, the FC binding sequence $V_{94}TK^+YWFYR^+$ was recently predicted for caveolin-1 (*42*). Comparable FC binding motifs have been identified in the STAR, NPC2, and Nef proteins (*43*−*45*). If the function of this sequence within caveolin-1 is borne out by additional mutagenesis, the role for S_{80} suggested above and the interaction between the FC binding site and Y_{14} suggested above imply the presence of significant caveolin-1 tertiary structure in caveolae.

Effects of PDGF on Caveolin-Associated Sterol. Under several experimental conditions, p(Y_{14})-caveolin-1 levels generated in response to PDGF were inversely related to the amount of caveolin-1-associated FC. Specifically, 30 min following the addition of PDGF, decay in p(Y_{14})-caveolin-1 levels was accompanied by recovery of FC. Inhibitors of PDGFR and PKC inhibited both p(Y_{14})-caveolin synthesis and loss of FC from caveolin-1. Glucose oxidase, an inhibitor of LMW-PTP, reduced both the decay of p(Y_{14})-caveolin-1 levels after PDGF and the restoration of FC levels in caveolae. p(Y_{14})-caveolin-1 bound sterol to a significantly lower extent than did the unmodified protein. Further experiments showed caveolin-1 Y_{14} phosphorylation to be not the cause but a consequence of the loss of sterol from caveolin-1. Finally, sterol was lost in the presence of PDGF from mutant (Y14A)caveolin-1, which lacks the reactive kinase site, just as it was from the wild-type protein.

The effect of PDGF on signal transduction via PDGFR is to transiently modify the phosphorylation on downstream signaling proteins also associated with caveolae (*4, 5*) such as the linker protein Shc and c-Src. These proteins copurify and are recovered in purified preparations of caveolae and contain consensus sequences for caveolin-1 binding (*2*). We suggest that the association of these phosphoproteins with the central domain of caveolin increases the displacement of sterol from caveolin-1 during signal transduction.

Roles for p(Y_{14})-caveolin-1 in the response to apoptosis and also in caveolin aggregation have been described (*46, 47*). The finding here that glucose oxidase blocked relipidation of caveolin-1 showed that phosphocaveolin-1 hydrolysis was needed for the recovery of FC binding. An additional function for p(Y_{14})-caveolin-1 can now be suggested: to regulate the magnitude and duration of the response to growth factors at this site by inhibiting FC binding. Overall, the data in this study suggest that, both in the resting state and following exposure to PDGF, S/T- and Y-phosphorylation can regulate FC binding and dynamically control the initial loss and subsequent recovery of sterol by caveolae.

Caveolin-1 Sterol Content and FC Efflux. SMC express large numbers of caveolae (*48*). The cell cycle is initiated in the presence of polypeptide growth factors including PDGF. In contrast, continuous and immortalized cells, like many cancer cell lines, are independent of growth factors and express few if any caveolae (*49*). SMC responded to PDGF with a 2-fold increase in efflux rates (*18*) over a time course that paralleled the synthesis of p(Y_{14})-caveolin-1 and the loss of caveolin-associated FC. In the present study, comparison of apo A-1-dependent efflux rates and decrease in caveolin-1-associated FC under different conditions provided further evidence that, at least in SMC, caveolae were a source of FC lost from the cell. The effects of inhibitors on caveolar FC, and on both basal and activated FC efflux rates, were almost identical. In each case, rates were inhibited by AG1296 and NGIC-I but resistant to PP2. This consistent pattern is suggestive of a caveolar origin for a significant proportion of both basal and PDGF-activated FC efflux in these cells. In this study, caveolin-1-associated FC represented only a small fraction of total cell sterol. However, the recovery of caveolar FC levels after PDGF illustrates that this can be readily replaced from intracellular pools. FC-enriched domains in synthetic membranes and in caveolin-free lipid rafts in cells are in the liquid-ordered state (*2*). Further research will be needed to identify the physical state of FC in caveolae, which is distinguished by the labile interaction between FC and caveolin-1 shown in this study.

Low levels of p(Y_{14})-caveolin were always detected even in serum-starved cells. The ligand-independent activation of extracellular receptor kinases (for example, by lysophosphatidic acid) has recently been described (*50*) and may explain this finding. Our data are consistent with the conclusion that both basal and activated FC efflux from primary SMC may originate from caveolae. Of course, this effect is likely to be less in cells with fewer caveolae, including many transformed cell lines (*49*).

In summary, this research provides further evidence for a dynamic relationship between FC and signaling in caveolae, suggests a molecular basis for this relationship, and could provide a foundation for future analyses of structure−function relationships within caveolin.

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